ACTA PHYSIOLOGICA SCANDINAVICA
Vol. 44. Supplementum 151

FROM THE INSTITUTE OF PHYSIOLOGY, UNIVERSITY OF HELSINKI, FINLAND

BEHAVIOUR OF PYRUVIC ACID IN PLASMA AND RED CELLS IN RABBITS BLED REPEATEDLY

By

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VAMMALA 1958

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I. Introduction.

Interest in this problem was aroused in this laboratory when various blood constituents were being analysed during the period of accelerated blood regeneration due to repeated bleedings. The results regarding the concentration changes of ascorbic acid, total free amino acids and reducing sugars of the plasma and red cells have already been reported (*Piha* 1956). The experiments were partly designed to reveal whether the chemical changes found during progressive reticulocytosis were possibly involved in haemoglobin synthesis. Since pyruvic acid occupies a central position in intermediary metabolism, one would expect that there might also be changes in the concentration of pyruvate in the blood after repeated bleedings.

A number of reports have been made on the effect of a deficient supply of oxygen on the pyruvic acid content of the blood. Under conditions characterized by lack of oxygen, a considerable part of the energy is derived from anaerobic metabolism, in which large amounts of organic acids are formed. Such conditions are found under reduced atmospheric pressure, in haemorrhagic and haemolytic anaemias, in foetal life and immediately after birth, in severe heart disorders and in carbon monoxide poisoning. Furthermore, such conditions are sufficient to stimulate accelerated production of red cells and haemoglobin. An increased percentage of reticulocytes is known to be a distinctive feature in these conditions.

Though a wide variety of anoxic conditions have been investigated, very little attention has been paid to changes in the pyruvic acid metabolism during accelerated blood regeneration, particularly when due to repeated bleedings. The present work deals with the changes in concentration of pyruvic acid in the

plasma and red cells in the anaemia produced by repeated bleedings and during the subsequent recovery period in the rabbit. The relationship of the pyruvic acid content to the reticulocytosis is then dealt with. In order to elucidate the distribution of pyruvic acid between red cells and reticulocytes the ratio between red cell and plasma pyruvic acid is examined. The effect on the behaviour of pyruvic acid of the different plasma protein levels concurrent with haemorrhagic anaemia has also been studied. Some of the results have already been communicated (*Lähdevirta, Piha* and *Raina* 1957).

II. Survey of earlier investigations.

The concentration changes of blood pyruvic acid in various conditions and disorders has been the subject of a vast number of investigations. This survey is concerned only with its behaviour in conditions associated with a deficient supply of oxygen. An exhaustive review on pyruvic acid metabolism, viewing matters especially from clinical aspects, has recently been given by *Schreier* (1956).

The *normal concentration* of pyruvic acid in the blood varies to some extent, depending on species. Average blood pyruvate values from 0.55 to 1.09 mg/100 ml in adult man have been reported (*Lu* 1939, *Platt* and *Lu* 1939, *Wortis, Goodhart* and *Bueding* 1941, *Bueding, Wortis* and *Stern* 1942, *Klein* 1942, *Yanof* 1942, *Golberg* and *Gillman* 1943, *Droese* 1944, *Davis* and *Bauer* 1944, *Friedemann, Haugen* and *Kmieciak* 1945, *Goldsmith* 1948, *Kirk* and *Chieffi* 1949, *Markees* and *Meyer* 1949, *Amatuzio* and *Nesbitt* 1950, *Havel* and *Watkins* 1950, *Markees* 1950, *Markees, Käser* and *Lanz* 1950, *Tallqvist* 1952, *Lasch* 1953, *Friedberg* and *Diemer* 1954, *Antalóczy, Bretán* and *Lövei* 1956, *Redetzki, Bloedorn* and *Bansi* 1956, *Schreier* 1956, *Kyank* 1957, *Gonzales* and *Gardner* 1957). A lower value of 0.43 mg/100 ml has been obtained by *Gastaldi* (1955), using a chromatographic method. *Hess* (1955) has determined an average con-

centration of 0.40 mg for serum and *Henley, Wiggins* and *Pollard* (1956) 0.69 mg per 100 ml for plasma, both applying an enzymatic estimation technique. Figures for the normal blood pyruvic acid in children, apart from newborn infants, do not differ essentially from those of adults (*Kato* and *Li* 1941, *Wortis, Goodhart* and *Bueding* 1941, *Allibone* and *Finch* 1946, *Kirk* and *Chieffi* 1949, *Schreier* 1949, 1956, *Passweg* 1950, *Schreier* and *Dankwart* 1951, *Segagni* and *Bono* 1955).

The blood of the healthy animals studied exhibits a higher concentration of pyruvic acid than human blood. For instance, in the dog average values from 1.09 to 2.0 mg/100 ml (*Flock, Bollman* and *Mann* 1938, *Govier* and *Greer* 1941, *Beatty* 1945, *Greene* and *Phillips* 1957), in the sheep from 2.1 to 4.4 mg/100 ml (*Austin* 1947, *Karvonen* et al. 1949, *Brandt, Harned* and *Cooke* 1958), in the rat from 0.98 to 1.8 mg/100 ml (*Lu* 1939, *v. Euler, Säberg* and *Högberg* 1941, *Engel, Winton* and *Long* 1943, *McShan* et al. 1945, *Frohman, Orten* and *Smith* 1951) and in the rabbit from 0.98 to 1.47 mg/100 ml (*Lu* 1939, *Markees* and *Meyer* 1949, *Vitacca* and *Pagano* 1955) have been found. The mean resting pyruvate in dog plasma is, according to *Huckabee* (1956), 0.221 mM/l of blood water.

Hitherto, pyruvic acid changes associated with *haemorrhage* have been studied only in short-term experiments, lasting in most cases between three and five hours. In this way a progressive elevation of the blood pyruvate concentration has been established in haemorrhagic dogs (*Govier* and *Greer* 1941), in fasted haemorrhagic rats (*Engel, Winton* and *Long* 1943) and in eviscerated rats as shock develops after taking blood to 1 1/2—2 per cent of body weight (*Russell, Long* and *Engel* 1944). The increase has been attributed to the anoxia attendant on the circulatory failure (*Engel* 1952). The increase is seen in rats after non-fatal and fatal haemorrhage (*Beatty* 1945, *Wilhelmi* and *Long* 1948). *Beatty* (1945) found a correlation between the severity of the haemorrhage and the rise of pyruvic acid. The smaller the amount of blood in the circulation, the greater was the rise of blood pyruvate. *Root* et al. (1947) found an increase of pyruvic acid in the plasma of dogs when their blood was reduced by haemorrhage to 60—70 per cent of the original

volume. The above metabolic changes seem to be associated with a state of insufficient oxygen supply, which depresses tissue oxidations, and with a secretion of epinephrine, which increases pyruvic acid (*Schreier* 1956, p. 89).

In *haemolytic* conditions raised pyruvic acid values have been found. *Allibone* (1948) showed that the blood pyruvate content in infants with haemolytic anaemia varied between 1.85 and 3.50 mg/100 ml, but fell after a blood transfusion. The level rose again immediately in the cases where the haemolytic process persisted. This phenomenon is in his opinion unrelated to the deficiency of thiamine. An increase of pyruvic acid in blood was recorded by *Lasch* (1953) in a patient with pernicious anaemia, but not in anaemia due to the lack of iron (3 cases). In haemorrhagic disease of the newborn abnormally high pyruvate values have also been found (*Kato* and *Li 1941*).

Some reports have been made on the effect of *simulated high altitudes* and *high mountains* on the pyruvic acid level in the blood. *Leipert* and *Kellersmann* (1942) found in glucose tolerance experiments a considerable elevation of blood pyruvate in male persons exposed to hypoxia. *Friedemann, Haugen* and *Kmieciak* (1945) and *Friedemann* et al. (1949) described an increase in the level on blood pyruvate in experiments when human subjects were allowed to breathe air under reduced barometric pressure at simulated altitudes above 4500 m. At elevated altitudes the rise of pyruvic acid was related to the degree of anoxia and to the length of exposure to anoxia. The rise was prevented or abolished by administration of oxygen. *Tepperman* and *Tepperman* (1948) established a corresponding pyruvate response to hypoxia after exercise in well-trained human individuals. *Monge* et al. (1948) and *Monge Cassinelli* (1949) studied the changes in blood pyruvate in persons living at sea level and on high mountains. At altitudes of 150, 3260, 3700 and 4540 m above sea level the corresponding average blood pyruvic acid values were: normal, 1.52, 2.16 and 2.13 mg/100 ml, respectively. The transfer of subjects from high altitudes to 150 m diminished the pyruvate content temporarily below, and after four months permanently to the normal value.

A number of investigations on pyruvic acid have been carried out using blood from the *newborn*, in whose tissues anaerobic metabolism preponderates over aerobic. Normal newborn infants have a raised blood pyruvic acid level (about 2 mg/100 ml), which stepwisely decreases (*Allibone* 1948) until at the end of the first week it equals the average value for healthy adults (*Gonzales* and *Gardner* 1957). Similar results were recorded by *Tallqvist* (1952), who found especially high pyruvate values in premature infants. *Schreier* and *Dankwart* (1951) demonstrated a temporary increase in the blood pyruvic acid up to an average value of 3.4 mg/100 ml during the first five hours following birth.

A discrepancy can be found between the results of the experiments carried out under reduced atmospheric pressure and in the newborn and those obtained by *Brandt*, *Harned* and *Cooke* (1958). The authors established a significant fall, from 1.7—3.7 to 1.0—1.8 mg/100 ml, in the concentration of blood pyruvic acid in progressive anoxia in a newborn lamb delivered by hysterotomy. The anoxia was produced by obstruction of the respiratory tract and clamping the umbilical cord immediately after birth. The results of *Greene* and *Phillips* (1957) obtained from experiments with hypoxic dogs agree with the above observation. Hypoxia induced by administration of oxygen-deficient air mixture to the ewe exerted, according to *Barker* and *Britton* (1958), no regular effect on the foetal blood pyruvate. The pyruvic acid fall is in agreement with a finding of *Ikeziri* (1956), who incubated bone marrow at low oxygen tensions. He found that at 16 per cent oxygen pyruvic acid production was distinctly increased, but at 6 per cent slightly decreased and at 1 per cent markedly so.

Schreier (1956) has reported that *umbilical cord* blood contains three times as much pyruvic acid as the blood of infants and children. The pyruvic acid level in the umbilical artery is slightly higher than that in the umbilical vein (*Karvonen* et al. 1949, *Tallqvist* 1952, *Gonzales* and *Gardner* 1957). The umbilical artery carries blood from the foetus to the placenta. According to *Gonzales* and *Gardner* (1957), during childbirth the pyruvate content in the umbilical arterial blood is somewhat

higher than that in the maternal blood. They are of the opinion that the high content of pyruvic acid in the newborn subsequent to birth is of foetal rather than maternal origin. In contrast, *Kyank* (1957) believes that the relatively high pyruvate concentration in umbilical blood found at the end of delivery is due to diffusion from maternal blood, the elevated content of its pyruvate being thus a result of labour.

The increased pyruvic acid level in the umbilical cord blood and in the blood of the newborn may be associated with the relative anoxia which prevails during the period *in utero* and immediately after birth.

Some workers have studied pyruvate behaviour in states where *anoxia due to heart failure* is encountered. The findings of *Räihä* and *Tallqvist* (1949), *Havel* and *Watkins* (1950) and *Schreier* (1956) indicate an increased pyruvic acid content in the blood of children with congenital heart disease. *Segagni* and *Bono* (1955) could not confirm their results. In adults with decompensated heart failure, high blood or plasma pyruvate values have been reported by *Yanof* (1942), *Dalla Torre* (1952), *Antalóczy, Bretán* and *Lövei* (1956) and *Greif, Hochegger* and *Bermani* (1957), but not by *Goldsmith* (1948). The elevation is correlated with the degree of heart failure (*Yanof* 1942, *Greif, Hochegger* and *Bermani* 1957). According to *Räihä* and *Tallqvist* (1949) the increased pyruvate values are connected with anoxia of the tissues and inadequate oxygenation of the blood.

An interesting experiment was carried out by *Vitacca* and *Pagano* (1955), who treated rabbits repeatedly with *carbon monoxide* in order to induce experimental *hypoxia ad mortem*. They found that the pyruvic acid increases gradually, the average values of ten animals at the beginning of the experiment and on the 4th, 8th, 12th and 16th days after being 1.47, 3.15, 4.55, 4.60 and 4.90 mg/100 ml, respectively. The authors are of the opinion that the increase is connected with an altered carbohydrate metabolism resulting from a decreased oxygen supply.

Increased pyruvic acid values in the blood have also been reported after physical exercise (*Lu* 1939, *Lu* and *Platt* 1939, *Friedemann* and *Barborka* 1941, *Yanof* 1943, *Droese* 1944, *Tep-*

perman and *Tepperman* 1948, *Asmussen* 1950) and after operation (*Picco* and *Dogliotti* 1950, *Palomba* and *Budroni* 1952). The rise in pyruvic acid has likewise been ascribed to insufficiency of the oxidative processes due to poor oxygen delivery to the tissues (*Friedemann* and *Barborka* 1941, *Asmussen* 1950, *Palomba* and *Budroni* 1952).

From the foregoing, the general view emerges that increased pyruvic acid values are connected with conditions involving a deficient supply of oxygen. The increase seems to be correlated with the degree of the deficiency or inefficacy. No adequate explanation for the mechanism of pyruvate accumulation has been advanced because of the numerous factors involved.

III. Methods.

1. Animals used and diets.

Male and female rabbits of 3.0—5.0 kg body weight, 17 in number, were used for these experiments. A diet consisting of hay supplemented with green vegetable was given. Food and water were supplied *ad libitum*.

2. Performance of long-term experiments.

Anaemia was produced by removing blood from the marginal ear vein in amounts varying from 0.6 to 1.5 per cent of body weight (average 1.2 per cent) on four successive days. In a rabbit group, referred to as »*anaemic-hypoproteinaemic*», the blood volume lost was replaced with the corresponding volume of 0.9 per cent NaCl (saline). In rabbit group, referred to as »*anaemic-normoproteinaemic*», the blood volume removed was substituted by transferring back the autonomous plasma and the required volume of saline. Autonomous plasma and roughly an equal volume of excessive foreign plasma were injected intravenously into the rabbits of a group referred to as »*anaemic-hyperproteinaemic*». The transfusions were performed about three hours

after the bleedings. In certain experiments the rabbit was bled 5—6 times successively, every other day for a period of 7—9 days. This rabbit group is referred to as »*anaemic*» only. After treatment the animals were allowed to recover.

For analysis, blood samples of 4—6 ml were taken on the big bleeding days, collecting the initial flow of blood, and again at intervals during the course of the following 3—4 weeks, heparin (0.2 mg/ml of blood) being used as anticoagulant. Immediately after taking the blood it was cooled to 3—4° C, followed by centrifugation for 25 minutes at 3500 r.p.m. The plasma was then removed from the red cells.

3. Preparation of protein-free solutions.

The precipitation of proteins for pyruvic acid estimations was carried out using a 10 per sent solution of trichloroacetic acid. In order to avoid the conversion and the breakdown of pyruvic acid the protein precipitation was performed as soon as possible after centrifuging the blood. To prevent enzymatic action in another series of experiments, the blood was drawn into a syringe containing sodium iodoacetate in the proportion of 25 mg per 5 ml of sample, followed by immediate centrifugation of the blood and precipitation of the proteins.

The proteins of the plasma were precipitated by adding slowly, while stirring, 1 volume of plasma to 4 volumes of the 10 per cent trichloroacetic acid. After thorough shaking and allowing the mixture to stand for 10 minutes, the precipitate was centrifuged without delay for 25 min. at 3500 r.p.m. The clear supernatant liquid, dilution 1: 5, was collected. Before the precipitation of the red cell proteins, the cells were haemolysed with an equal volume of distilled water. The centrifugates thus obtained corresponded to a 1: 10 dilution of the red cell sample.

If immediate analyses were impossible, the protein-free solutions were stored in a refrigerator at 2° C. After addition of a few drops of toluene, pyruvic acid was found to keep unaltered at least for a week. To check this, fifteen samples were reanalysed until the 23rd—41st days.

4. Analytical methods.

Two identical samples were estimated in each instances, and the mean result was taken.

i. Number of erythrocytes.

For the red blood cell counts a *Levy* counting chamber was used, five large squares in two different chambers being counted in each determination. In the normal rabbits the number of erythrocytes varied from 5.2 to 6.0 millions/μl.

ii. Percentage of reticulocytes.

The reticulocytes were stained with brilliant cresyl blue and counted in dry preparations as described in detail in an earlier publication (*Piha* 1956). The number of reticulocytes was recorded until the total count reached 1000. The percentage of reticulocytes for the rabbits in normal conditions varied from 3.3 to 4.8 per cent.

iii. Volume of packed red cells (haematocrit).

Haematocrit determinations were made using graduated tubes with centrifugation for 25 minutes at 3500 r.p.m. No correction factor for trapped plasma was used.

iv. Haemoglobin.

Haemoglobin estimations were made immediately after taking the blood sample by a modification of the acid haematin method with a Beckman spectrophotometer, model DU, at a wavelength of 520 mμ.

v. Total plasma protein.

Total plasma protein concentration was determined by means of a modified method of *Gornall, Bardawill* and *David* (1949). 0.10 ml of clear plasma was mixed with 1.9 ml of 0.9 per cent

saline followed by the addition of 8 ml of biuret reagent*). After thorough swirling the mixture was allowed to stand for 30 minutes. The extinction was measured with a Beckman spectrophotometer at a wavelength of 540 mμ against a reagent blank. The standard error of a single determination calculated from 30 double estimations was found to be 1.8 per cent.

vi. Pyruvic acid.

Pyruvic acid was estimated from plasma and the red cells by a modification of the *Friedemann* and *Haugen* method (1943) as developed by *Markees* (1951). In the procedure pyruvic acid is converted to the dinitrophenylhydrazone derivative which is separated from other hydrazones with toluene followed by extraction with sodium carbonate solution. With sodium hydroxide solution the hydrazone gives a red colour. 3 ml of trichloroacetic acid supernatant was placed in a boiling water bath for 5 minutes in order to destroy any acetoacetic acid that may have been present,and then cooled. To the centrifugate 1 ml of 0.1 per cent 2,4-dinitrophenylhydrazine was added, and allowed to react for 5 minutes. The hydrazone formed was extracted with 3 ml of toluene by vigorous shaking of the glass-stoppered tube for 1 minute. After this, the lower aqueous layer was removed and the remaining mixture was shaken vigorously with 6 ml of 10 per cent sodium carbonate solution for 45 seconds. The two phases were allowed to separate. 4 ml of the lower layer was then transferred to a test tube and an equal volume of 1.5 N sodium hydroxide solution was added. When the contents of the tube were thoroughly mixed the absorption of light was read with a Beckman spectrophotometer at a wavelength of 520 mμ. The standard error of a single estimation was found to be 0.8 per cent for plasma and 2.7 per cent for red cells when calculated from 30 and 37 arbitrarily chosen duplicate determinations, respectively. A standard curve used was made with redistilled pyruvic acid (B.D.H. Laboratory reagent).

*) Biuret reagent: 1.5 g of cupric sulphate and 6.0 g of sodium potassium tartrate are dissolved in 500 ml of water. 300 ml of 10 per cent sodium hydroxide is added by swirling. The volume is made up to 1000 ml with water.

IV. Results.

In all, seventeen rabbits made anaemic by repeated bleedings were used for these experiments. The development of anaemia and hypo-/hyperproteinaemia in response to pretreatments is seen in Table I.

The haemoglobin content was reduced to 35—65 per cent of the initial value, the average being 43.8 per cent in the anaemic-hypoproteinaemic (Group I), 43.3 per cent in the anaemic-normoproteinaemic (Group II), 37.7 per cent in the anaemic-hyperproteinaemic (Group III) and 66.4 per cent in the anaemic (Group IV) groups. In Groups I, II and III a serious reduction of haemoglobin level is observed immediately after the first bleeding of about 1.3 per cent of body weight and the minimum is found on the third day of the experiment. Haemoglobin content begins to rise on the fifth day and the normal level is reached again on the 24th day. It is found that the development of anaemia and the recovery in the three first-mentioned rabbit groups is fairly similar. In Group IV, in which the rabbits were bled in amount of about 0.6 per cent of body weight every other day, the minimum percentage of haemoglobin is discovered on the 8th day.

The pretreatment altered the plasma protein levels to average values of 82.9, 97.0 and 112.1 per cent of the initial values in Groups I, II and III, respectively. In experimental anaemia-hypoproteinaemia the minimum plasma protein content was found on the third day. A distinct hyperproteinaemia seemed to be more difficult to induce in spite of the large volume of excessive plasma (about 40 ml of plasma/kg body wt. corresponding with about 2.5 g of plasma protein/kg) transfused into the circulation. However, only a relatively slight rise of plasma protein level was recorded, the maximum concentration being found on the 5th day. This may be partly explained by the increase of the plasma volume. The hypoproteinaemic state continues only until the 5th day, while the hyperproteinaemic state lasts to the 8th—12th day of the experiment.

Table I.

Course of anaemia and hypo-/hyperproteinaemia in response to pretreatments and the recovery.

Rabbit Group referred to as	Day of experiment	Blood volume withdrawn ml/kg	Plasma volume injected back ml/kg	Haematocrit value	Haemoglobin g/100 ml	Haemoglobin as percentage of the initial	Plasma protein g/100 ml	Plasma protein as percentage of the initial
Anaemic-hypo-proteinaemic (Group I) n = 4	Initial	12.8	—	38.2	13.1	100.0	6.68	100.0
	1	13.3	—	28.7	9.7	74.0	6.11	91.6
	2	13.0	—	20.9	6.9	52.8	5.77	86.5
	3	5.9	—	18.1	5.7	43.8	5.53	82.9
Total		45.0						
	5			20.6	6.9	52.8	6.48	97.0
	8			30.0	8.8	67.1	6.80	101.6
	12			35.7	11.2	85.9	6.71	100.2
	15			36.9	11.6	88.6	6.48	97.1
	24			38.5	12.6	96.3	6.91	103.2
	30			39.5	13.2	100.1	6.71	100.2
Anaemic-normo-proteinaemic (Group II) n = 4	Initial	11.8	6.1	38.0	12.8	100.0	7.01	100.0
	1	12.4	8.2	31.6	9.5	74.1	6.90	98.5
	2	12.0	8.2	22.0	7.0	54.6	6.84	97.5
	3	6.9	4.9	18.4	5.5	43.3	6.80	97.0
Total		43.1	27.4					
	5			18.9	6.4	50.5	7.20	102.4
	8			27.4	8.3	64.9	7.16	102.0
	12			35.9	10.6	82.4	7.26	103.4
	15			37.0	11.4	89.7	7.30	104.0
	24			40.6	13.4	104.5	7.17	102.0
	30			38.4	12.1	95.2	6.74	96.0
Anaemic-hyper-proteinaemic (Group III) n = 4	Initial	13.8	12.0	37.6	13.0	100.0	6.52	100.0
	1	15.1	23.6	28.2	9.2	70.4	6.45	99.0
	2	14.6	21.6	19.2	5.9	44.8	6.74	103.1
	3	7.8	9.7	16.2	4.9	37.7	6.87	105.2
Total		51.3	66.9					
	5			18.3	6.2	47.5	7.32	112.1
	8			25.3	8.1	61.6	7.04	108.0
	12			32.6	9.6	73.3	6.74	103.0
	15			35.4	10.8	82.6	6.66	102.0
	24			39.4	12.2	93.8	6.46	99.2
	30			41.3	12.5	96.0	6.48	99.5
Anaemic (Group IV) n = 5	Initial	6.1	—	38.2	10.8	100.0	—	—
	2	6.5	—	31.2	8.5	78.1	—	—
	4	5.4	—	27.2	8.0	73.6	—	—
	6	6.1	—	26.9	7.6	69.8	—	—
	8	5.8	—	27.2	7.2	66.4	—	—
	10	5.7	—	28.5	7.6	70.0	—	—
Total		35.6						
	12			—	8.5	78.5	—	—
	15			—	10.0	92.5	—	—
	20			36.6	10.1	93.7	—	—
	Final			37.2	10.5	97.3	—	—

Table II.

Effect of blood loss on the concentration of pyruvic acid in the plasma and the red cells.

Group No.	Rabbit No.	Plasma pyruvic acid mg/100 ml			Red cell pyruvic acid mg/100 ml			Pyruvic acid increase as percentage of initial	
		Initial value	Maximum value	Recovered value	Initial value	Maximum value	Recovered value	Plasma	Red cells
	Anaemic-hypoproteinaemic								
I	C—1	2.35	4.49	2.21	1.88	3.67	1.75	190	195
	R—1	2.66	5.89	2.80	2.11	4.29	1.70	221	203
	S—3	3.59	6.75	3.23	2.24	4.36	1.89	188	194
	Mean	2.86	5.71	2.75	2.08	4.11	1.78	200	197
	Anaemic-normoproteinaemic								
II	H—1	4.53	8.22	4.42	2.54	5.26	2.12	181	207
	U—1	1.78	3.82	2.02	1.62	3.94	1.64	214	243
	A—1	2.22	8.55[2])	2.82	1.50	4.07	2.04	384[2])	270
	Mean	2.84	6.86	3.09	1.89	4.42	1.93	260[3])	240
	Anaemic-hyperproteinaemic								
III	N—1	2.72	5.18	2.36	1.38	4.07	1.24	190	294
	F—1	2.73	4.40	—[4])	2.16	2.46	—[4])	161	114
	S—2	1.31	2.10	1.24	1.73	3.25	1.78	160	188
	Mean	2.25	3.89	1.80	1.76	3.26	1.51	170[5])	199[5])
	Anaemic [1])								
IV	215—3	4.71	5.95	5.57[6])	3.11	4.64	3.44	126	149
	277—4	4.85	6.94	4.64	2.98	5.54	2.77	143	186
	OM—6	4.36	5.85	4.83	3.13	5.07	2.75	134	162
	Mean	4.64	6.25	5.01	3.07	5.08	2.98	134	166
Mean of the rabbits of Groups I-IV		3.14	5.68	3.00	2.09	4.22	2.03		
Mean of the rabbits of Groups I-III		2.66	5.49	2.64	1.91	3.93	1.77		

[1]) Iodoacetic acid added immediately after taking the blood sample. [2]) The value seems to be too high. [3]) The mean value is 198, the value 384 excluded. [4]) The rabbit died after the bleedings. [5]) The mean values are 175 and 241, the values 161 and 114 (erroneous?) excluded, respectively. [6]) The rabbit had not recovered.

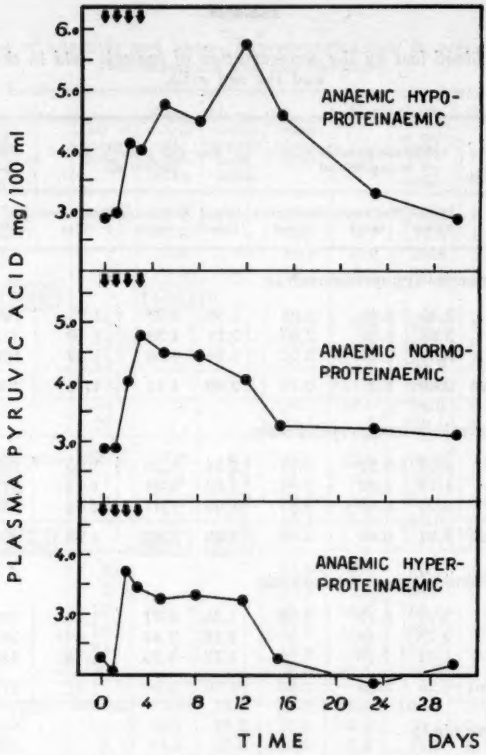

Fig. 1. Effect of repeated bleedings on the pyruvic acid concentration of plasma in anaemic-hypoproteinaemic, anaemic-normoproteinanaemic and anaemic-hyperproteinaemic rabbit groups. The bleeding days are marked by arrows.

1. Changes in the pyruvic acid content of the plasma and red cells in response to repeated bleedings.

The effect of repeated bleedings on plasma and the red cell pyruvate are seen in Table II and Figs. 1 and 2.

Table II shows the initial, maximum and recovered values of pyruvic acid in the anaemic-hypoproteinaemic, anaemic-normoproteinaemic, anaemic-hyperproteinaemic and anaemic groups.

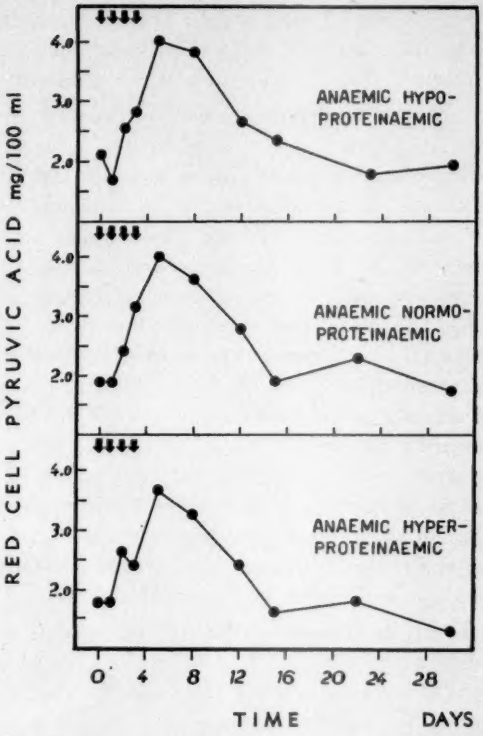

Fig. 2. Effect of repeated bleedings on the pyruvic acid concentration of red cells in anaemic-hypoproteinaemic, anaemic-normoproteinaemic and anaemic-hyperproteinaemic groups. The bleeding days are marked by arrows.

As is evident from the table, the concentration of pyruvate increases conspicuously in both plasma and the red cells. The maximum increases are found after the 3rd—4th bleeding and the average values, expressed as a percentage, exceed the initial *plasma* values, estimated as 2.9, 2.8, 2.3 and 4.6 mg/100 ml, by 100, 160, 70 and 34 per cent in Groups I, II, III and IV, respectively. The corresponding data of the *red cells* are: the maximum increases 97, 140, 99 and 66 per cent, the initial values

being 2.1, 1.9, 1.8 and 3.1 mg/100 ml. The relatively high initial values for pyruvic acid in Group IV are not comparable with those of Groups I, II and III because of the different analytical technique. Iodoacetic acid was used for blood samples of Group IV.

Fig. 1 illustrates changes of *plasma pyruvic acid* in anaemic-hypoproteinaemic, anaemic-normoproteinaemic and anaemic-hyperproteinaemic groups, during the 30-day experimental period. The days of large bleedings are marked by arrows. From the pyruvic acid curves it appears that as a result of the bleedings the plasma pyruvic acid increases. In the anaemic-hypoproteinaemic group it rises up to the twelfth day, the increase being about 100 per cent. In the anaemic-normoproteinaemic and anaemic-hyperproteinaemic groups the pyruvic acid increase occurs suddenly the average maximum of about 65 per cent being already reached on the third day of the experiment. Thereafter the pyruvate content keeps fairly high up to the twelfth day. In each group pyruvic acid begins to fall after the twelfth day and the normal levels are reached in Group I within about two weeks and earlier in Groups II and III.

Fig. 2 shows the changes of the *red cell pyruvic acid* in the corresponding rabbit groups. It is to be observed that, like the development of anaemia and the recovery, the red cell pyruvic acid curves of the anaemic-hypoproteinaemic, anaemic-normoproteinaemic and anaemic-hyperproteinaemic groups are all similar. The maximum concentration is reached in each instance on the fifth day of the experiment, the increase being about 100 per cent. The pyruvate content then falls steadily and the approximate initial level is reached on the fifteenth day.

By comparing the plasma pyruvate curves (Fig. 1) with the red cell pyruvate curves (Fig. 2) it is seen that the shapes of the above graphs are roughly similar. The data of an individual rabbit (H-1) have been plotted against time in Fig. 3. It is observed that the concentration changes of the red cell pyruvate run almost parallel with those of the plasma pyruvate. The results indicate that the pyruvic acid concentration in the red cells depends partly on the plasma pyruvate concentration.

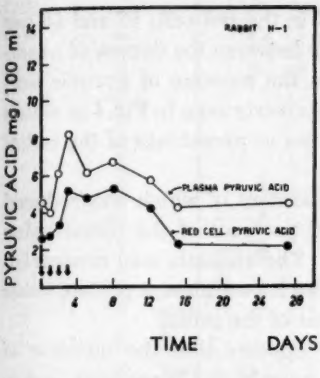

Fig. 3. Changes in the pyruvic acid concentration of the plasma and red cells as a function of time. Rabbit H-1. The bleeding days are marked by arrows.

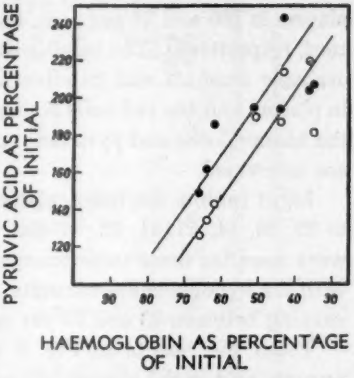

Fig. 4. Correlation between the maximum increase of pyruvic acid and the degree of haemorrhagic anaemia. Eight rabbits. ○ Plasma pyruvic acid. ● Red cell pyruvic acid.

To sum up, the average concentration of pyruvate in normal rabbit plasma is 2.65 (S.D. = 0.92) and the red cells 1.84 (S.D. = 0.33) mg/100 ml. In response to repeated bleedings its content increases in plasma by 34—160 per cent and in the red cells by 66—140 per cent depending on the state produced. The different plasma protein status does not seem to have any effect on the pyruvate increase in the red cells, whilst the rise of pyruvic acid in plasma is least in the anaemic-hyperprotein-aemic group. A parallelism between the changes of the pyruvic acid concentration in the plasma and the corresponding changes in the red cells is evidenced.

2. Correlation between the degree of haemorrhagic anaemia and the increase of pyruvic acid.

From Tables I and II it can be seen that as the haemoglobin content is reduced to 43.8 per cent (Group I) and 66.4 per cent (Group IV) of the initial level, the pyruvic acid increase in the

plasma is 100 and 34 per cent, and in the red cells 97 and 66 per cent, respectively. The relationship between the degree of haemorrhagic anaemia and its effect on the increase of pyruvic acid in plasma and the red cells is more clearly seen in Fig. 4 in which the haemoglobin and pyruvate values as percentage of the initial are expressed.

Eight rabbits the haemoglobin content of which was reduced to 35, 36, 44, 45, 51, 62, 64 and 66 per cent of the initial value were used for these experiments. The anaemia was concurrent with the hypoproteinaemic state, the lowest plasma protein value varying between 83 and 93 per cent of the initial.

From the results in Fig. 4 it appears that the increase of pyruvic acid in the plasma varies from 26 to 120 per cent, and in the red cells from 49 to 143 per cent. A general impression is gained that the more severe the deficiency of haemoglobin due to repeated bleedings the greater is the maximum increase of pyruvic acid in the plasma and red cells. It is revealed also that the points representing the pyruvate increases of the plasma are lower than the corresponding points of the red cells.

To sum up, a correlation between the degree of haemorrhagic anaemia and the increase of pyruvic acid in the plasma and red cells is evidenced.

3. Ratio between the red cell and plasma pyruvic acid as a function of time.

It is logical to think that if the ratio between the red cell and plasma pyruvic acid keeps constant the amount of pyruvic acid in red cells is a sole function of the pyruvic acid content of the plasma. It was observed that the changes of pyruvic acid in plasma and the red cells during the bleeding period are roughly similar. A more detailed consideration discloses, however, that a direct proportionality is not found. It is revealed clearly by the concentration ratio of pyruvic acid between the red cells and the plasma*). Tables III and IV give the ratios as a function of time and in response to a number of bleedings.

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*) [Red cell pyruvate] / [Plasma pyruvate], [Red cell PA] / [Plasma PA].

Table III.

Ratio of the concentrations of red cell pyruvic acid and plasma pyruvic acid during the course of the bleeding period and the recovery.

Rabbits bled (1.2 % of body wt.) on four successive days.

Rabbit Group referred to as	Concentration ratio [Red cell PA] / [Plasma PA]								
	Normal state	In response to				The recovery			Recovered state
		one bleeding	two bleedings	three bleedings	four bleedings				
	Initial day	1st day	2nd day	3rd day	5th day	8th day	12th day	15th day	24th—30th day
Anaemic-hypoproteinaemic (Group I)	0.74	0.60	0.67	0.74	0.88	0.89	0.48	0.57	0.67
Anaemic-normoproteinaemic (Group II)	0.71	0.63	0.63	0.53	0.90	0.80	0.65	0.59	0.67
Anaemic-hyperproteinaemic (Group III)	0.87[1])	0.80	0.89	0.88	1.29	1.13	0.93	0.88	0.73
[2]) Reticulocytes, per cent	2.1	2.8	5.0	12.3	17.3	—	—	—	4.8
Red blood cells, millions/μl	4.85	3.82	2.86	2.01	1.96	—	—	—	5.30
Haemoglobin, %:age of initial	100.0	79.1	59.6	47.3	52.9	—	—	—	102.0
Haematocrit value	38.2	28.7	20.9	18.1	20.6	30.0	35.7	36.9	38.0

1) The high ratio level averaged depends on the unusually high level of the rabbit S-2. The ratios are, S-2 values excluded: 0.65, 0.60, 0.68, 0.68, 1.10, 0.95, 0.74, 0.76, 0.54.

2) The data have been obtained from the experiments, 9 in number, corresponding to Group I (anaemic-hypoproteinaemic).

Table IV.

Ratio of the concentrations of red cell pyruvic acid and plasma pyruvic acid during the course of the bleeding period and the recovery.
Rabbits bled (0.6 % of body wt.) 5—6 times successively, every other day.

Rabbit Group referred to as	Concentration ratio [Red cell PA] / [Plasma PA]						
	Normal state	In response to					Recovered state
		one bleeding	two bleedings	three bleedings	four bleedings	five bleedings	
	Initial day	2nd day	4th day	6th day	8th day	10th day	17th—20th day
Anaemic (Group IV)	0.66	0.72	0.78	0.86	1.00	0.89	0.61
Reticulocytes, per cent	3.9	6.1	8.2	11.0	16.2	21.5	5.2
Red blood cells, millions/μl	5.48	4.46	3.82	3.72	3.42	3.67	5.09
Haemoglobin, %:age of initial	100.0	82.5	71.5	70.1	67.4	72.0	91.2
Haematocrit value	38.2	31.2	27.2	26.9	27.2	28.5	37.2

It is observed in Table III that in Groups I, II and III the concentration ratio [Red cell pyruvate] / [Plasma pyruvate] first diminishes from its initial values of 0.74, 0.71 and 0.87 (0.65) and keeps under the normal level during the time of the four successive bleeding days. Thereafter the ratio rises steeply in the anaemic-hypoproteinaemic group to a value of 0.89, in the anaemic-hypoproteinaemic group to a value of 0.89, in the anaemic-normoproteinaemic group to 0.90 and in the anaemic-hyperproteinaemic group to 1.29 (1.10), the maximum ratios being found on the fifth day. The ratio remains high until the 8th day and then falls fairly quickly. After restoration of the normal condition of the rabbit the original concentration ratios between red cells and plasma are found again.

Table IV gives the corresponding results of Group IV in which the rabbits were bled about 0.6 per cent of body weight every other day. In these experiments the reticulocytosis was allowed to progress gradually and the degree of anaemia did not become very severe. In this way an attempt was made to maintain balance in the animal body whereas this must be disturbed in

Groups I, II and III in consequence of the daily large bleedings. From the results it appears that the concentration ratio rises gradually from 0.66 up to a value of 1.00 found on the 8th day, in response to four bleedings. On comparing the ratios with the corresponding haematocrit values, it appears that there is no noticeable change in the volume of the packed red cells as the ratio increases from 0.78 (4th day) to 1.00 (8th day). Hence, the increase of the pyruvate ratio is not explained by a reduction of the total red cell volume.

The erythrocyte and haemoglobin values of Group IV which correspond to the highest [Red cell PA] / [Plasma PA] ratio are 3.42 millions/μl and 67.4 per cent of the initial value, respectively (Table IV). The corresponding figures for the everyday bleeding groups are 1.96 millions/μl and 52.9 per cent (Table III).

To sum up, these findings indicate that the passage of pyruvic acid into red cells is not solely a physico-chemical process. The [Red cell pyruvate] / [Plasma pyruvate] ratio changes. In response to four bleedings the ratio rises to maximum and remains high for some days.

4. Relationship of the [Red cell pyruvate] / [Plasma pyruvate] ratio and red cell pyruvic acid content to the reticulocyte percentage.

From Table III it appears that the initial ratio [Red cell PA] / [Plasma PA] of 0.74 corresponds to the reticulocyte value of 2.1 per cent. The corresponding figures in Group IV (Table IV) are 0.66 and 3.9. In response to four bleedings the mean reticulocyte counts of 17.3 per cent (Group I, 5th day) and 16.2 per cent (Group IV, 8th day) have been recorded. The corresponding ratios are 0.9 and 1.0.

The every-day bleeding technique of Groups I—III is not satisfactory in comparing the [Red cell PA] / [Plasma PA] to reticulocytosis because a considerable number of new reticulocytes is released from the bone marrow not until on the third day after the first bleeding. The reticulocytosis does not develop

Table V.

Relationship of the red cell pyruvic acid and the [Red cell pyruvate] / [Plasma pyruvate] ratio to the progressive reticulocytosis.

Rabbit	Blood picture				Reticulocyte percentage above the initial count	Red cell pyruvic acid as percentage of the initial	[Red cell PA] / [Plasma PA]
	Haemoglobin as percentage of the initial	Haematocrit value	Erythrocytes mill/μl	Reticulocytes per cent			
			Initial state				
OM-6	100	38.4	5.23	3.3	0	100	0.71
277-4	100	37.8	5.70	3.9	0	100	0.62
215-3	100	37.4	5.77	4.2	0	100	0.66
246-1	100	39.7	6.02	4.3	0	100	0.77
277-3	100	39.2	5.29	4.8	0	100	0.65
Mean	100	38.5	5.60	4.1	0	100	0.68
			Progressive reticulocytosis				
215-3	86.8	—	4.66	5.4	1.2	104	—
OM-6	81.8	27.8	4.43	4.6	1.3	97	0.65
277-4	90.9	31.4	4.64	5.8	1.9	93	0.70
246-1	77.6	26.2	3.67	7.4	3.1	93	0.80
215-3	73.6	26.2	4.05	7.3	3.1	107	0.64
277-4	69.5	26.0	3.81	7.2	3.3	108	0.85
215-3	78.0	—	4.17	9.7	5.5	127	0.72
OM-6	66.1	26.2	2.98	8.9	5.6	119	0.84
215-3	67.1	26.5	3.71	10.7	6.5	149	0.78
OM-6	68.0	25.1	3.35	10.5	7.2	160	0.86
277-4	68.7	27.5	3.36	12.7	8.8	131	0.96
277-4	65.1	26.8	3.25	14.8	10.9	152	1.07
215-3	72.6	27.6	3.93	16.3	12.1	138	0.75
OM-6	70.4	28.2	3.08	20.8	17.5	162	1.16
277-4	69.1	27.0	3.49	28.1	24.2	186	1.02
			Recovered state				
277-4	97.7	39.0	5.30	6.9	3.0	95	0.60
215-3	94.0	36.1	5.28	5.8	1.6	110	0.62
OM-6	89.2	34.4	4.45	3.4	0.1	88	0.57
Mean	93.6	36.5	5.01	5.4	1.6	97.7	0.60

gradually. For the above purpose the experiments, five in number, in which rabbits were bled every other day are more expedient. The results are seen in Table V and in Fig. 5, in which are presented also the changes of red cell pyruvic acid as percentage of the initial value.

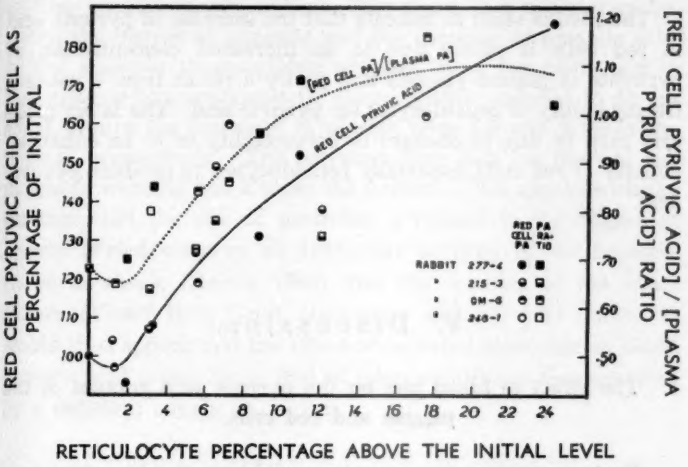

Fig. 5. Relationship of the red cell pyruvic acid and the [Red cell pyruvate] / [Plasma pyruvate] to the progressive reticulocytosis. The rabbits were blev every other day to about 0.6 per cent/kg body wt.

From the table it appears that during the progressive reticulocytosis from 4 to 21—28 per cent the concentration ratio rises from the average value of 0.68 to about 1.1. Individual variations in the ratio level between different rabbits can be found. Most clearly this is seen in Fig. 5, which shows also the relationship between the red cell pyruvic acid and the percentage of reticulocytes. It is revealed that pyruvic acid increases with the increasing number of reticulocytes. However, pyruvic acid content in red cells does not increase until the increase of reticulocyte count has passed two per cent. As the number of reticulocytes rises to some six, twelve and twenty two per cent above the initial level the corresponding rise of red cell pyruvate is about 25, 50 and 85 per cent in response to some 2, 3 and 4—5 bleedings, respectively. For instance, in the rabbit 277—4, on the 5th day of the experiment when the rabbit was bled three times, an increase of 31 per cent is observed, which corresponds to a 8.8 per cent rise in the number of reticulocytes.

The results seem to indicate that the increase of pyruvic acid in red cells is partly due to an increased concentration of pyruvate in plasma (blood) and partly a result from a concentrating ability of reticulocytes for pyruvic acid. The latter property may be due to changes in permeability or to an enhanced activity of red cells, especially reticulocytes, to produce pyruvic acid.

V. Discussion.

1. The effect of blood loss on the pyruvic acid content of the plasma and red cells.

There are only a few reports available in the literature concerning pyruvic acid changes in haemorrhage. The studies of *Engel, Winton* and *Long* (1943), *Russell, Long* and *Engel* (1944) and *Root* et al. (1947), who found an increase in blood pyruvate content after withdrawing 30—50 per cent of the blood, are not comparable, because the state produced was haemorrhagic shock and the experiments lasted only 1 to 5 hours. The present work gives the results of long-term experiments covering 20—30 days, the bleeding period lasting from 4 to 10 days. However, a similar increase was established in both plasma and red cells in rabbits bled repeatedly. From the normal mean level of 2.66 mg/100 ml in the plasma and 1.91 mg/100 ml in the red cells the pyruvic acid concentration rose to average values of 5.49 and 3.93 mg/100 ml, respectively. In short-term experiments in dogs subjected to a severe but not fatal haemorrhage, the average blood pyruvate four hours after bleeding rose from an initial value of 1.5 mg to 4.0 mg per 100 ml (*Beatty* 1945). A sharp rise was shown during the first hour following haemorrhage, whilst in the present investigation the steepest rise of pyruvic acid, especially in the red cells, occurred on the 3rd—5th day of the experiment. This period corresponds to the time required for the development of a new cell population to the reticulocyte stage (*Piha* 1956).

The present results indicate that there is a correlation between the degree of anaemia and the increase of pyruvic acid. In the anaemic (haemoglobin reduced to 66 per cent) and in the anaemic-hypoproteinaemic (haemoglobin reduced to 44 per cent) groups the increases in plasma were 1.3 and 2 times the normal, respectively. The corresponding data for the red cell pyruvate were 1.6 and 2 times the normal. This agrees with the findings that the rise of pyruvate is related to the degree of anoxia (*Friedemann* et al. 1949), the severity of the haemorrhage in shock (*Beatty* 1945) and the severity of the heart failure (*Yanof* 1942, *Greif, Hochegger* and *Bermani* 1957). It would thus appear that the effect of repeated bleedings on blood pyruvic acid is similar to that of other conditions characterized by a deficient supply of oxygen.

2. The effect of different plasma protein levels on plasma and red cell pyruvic acid in anaemia due to repeated bleedings.

The hypo- and hyperproteinaemias concurrent with the anaemia were not very far advanced. In the former case the concentration of total plasma protein showed a value of 83 per cent of the initial, whilst in the latter case a maximum value of 112 per cent of the initial was found. Both the difficulty in producing these states and the quick restoration of the normal plasma protein level may be due to the protein pool which is distributed between blood and lymph.

The effect of these hypo- and hyperproteinaemic states on blood pyruvate seems to be of minor importance. The results are not very conclusive. It can be shown, however, that in the anaemic-hypoproteinaemic group the value averaged from the maximum figures of pyruvate increase of each of the experiments is 100 per cent in the plasma and 97 per cent in the red cells (see Table II). The corresponding data in the anaemic-hyperproteinaemic group are 70 and 99 per cent. The maximum increases obtained from the curves expressed as a function of time (see Figs. 1 and 2) are 100 per cent in the plasma and 94

per cent in the red cells in the former group, whilst in the latter group the values are 64 and 108 per cent. Thus, the results appear to indicate that in haemorrhagic anaemia a higher plasma protein content reduces the rise in plasma pyruvic acid concentration.

The finding that the pyruvate rise in the plasma is more striking in the anaemic-hypoproteinaemic state tallies with the observation that in the newborn the low plasma protein content (*Pommerenke* 1936) is associated with a high blood pyruvate concentration (*Allibone* 1948, *Gonzales* and *Gardner* 1957). It may be assumed that through transamination reactions pyruvic acid participates in plasma protein metabolism.

3. Red cell pyruvic acid and distribution between red cells and reticulocytes.

The present results indicate that the red cell pyruvate curves as a function of time are very similar in form in the different groups and that the increase of red cell pyruvate is correlated with the percentage of reticulocytes. Hitherto, only *Allibone* (1948) has recorded reticulocyte counts with blood pyruvate, without, however, comparing these with each other. From his data obtained from four infants suffering from haemolytic anaemia it can be seen that pyruvate values of 3.50, 1.88, 2.35 and 2.50 mg/100 ml correspond to reticulocyte counts of 34.8, 14.6, 13.2 and 9.0 per cent, respectively.

Is the increment of pyruvic acid found during the progressive reticulocytosis equally distributed among all the circulating red cells or is this substance concentrated more in the reticulocytes than in the mature cells? A consideration of the [Red cell pyruvate] / [Plasma pyruvate] ratio might throw light on this problem.

During the course of the first two days of the experiment, there is only a slight increase in the number of reticulocytes, comprising the most mature stages. This increase, which is due

to the sudden loss of blood, coincides with a slight increase of the ratio, or in Groups I, II and III with a slight decrease of it. The population of reticulocytes entering the circulation after the third day contains more and more younger, newly formed, metabolically active forms. Simultaneously an increase in the ratio is found, the further increase taking place simultaneously with the gradual increase in the number of reticulocytes.

When twelve experiments are treated together and averages for the initial, maximum and recovered values of the concentration ratios are estimated, the following data are obtained:

Ratio	0.69	1.00	0.63
Standard deviation (S.D.)	0.12	0.18	0.10
Reticulocytes, %	3.0	16.4	5.0

The average concentration of pyruvate per unit volume is normally definitely lower in the red cells than in the plasma, but during the crisis of the reticulocytosis it becomes approximately the same. However, if expressed per unit of water in the cells (68—74 % of the total content) the normal ratio is around unity and the highest value 1.43. Disequilibrium between red cell and plasma pyruvate has been alleged earlier in dog by *Flock, Bollman* and *Mann* (1938).

From the above consideration the view seems plausible that all red cells are freely permeable to pyruvic acid, the content of which roughly depends on the pyruvate concentration of the whole blood (plasma), but that the young reticulated cells also possess the ability to concentrate pyruvic acid which makes possible to exceed the [Red cell pyruvate] / [Plasma pyruvate] ratio of unity. The latter property points to intracellular processes (for instance, increased formation of pyruvic acid), and/or to differences in permeability of the mature and reticulated cells to pyruvic acid. Reticulocytes of different age may behave towards pyruvic acid in different way. It seems also that the fall of pyruvic acid to the normal level is delayed in young red cells, even after the loss of the reticulum. These ideas are supported by the experiments of *Huckabee* (1956) on mammalian

erythrocytes. He showed that the concentration gradient of pyruvic acid between extracellular and intracellular water is closely related to the plasma pyruvate content throughout its wide range. He concluded that pyruvate enters the red cell partly by passive diffusion and partly by an active mechanism due to the metabolic activity of the cells.

4. Augmentation of pyruvic acid and deficiency of oxygen supply.

The interpretation of the raised pyruvic acid values is rendered difficult by the numerous factors involved. Metabolic changes in general associated with haemorrhage are the result of prolonged and progressive anoxia. However, in haemorrhagic shock the chemical changes immediately after blood loss are undoubtedly due to the secretion of epinephrine (*Wilhelmi* and *Long* 1948).

To elucidate the question of what is responsible for the increase of pyruvic acid in the blood after repeated blood tappings, the relationship between pyruvic acid and conditions with a deficient supply of oxygen must be considered. Severe haemorrhagic anaemia with resulting reduction of cellular oxygen tension is comparable with other states characterized by deficiency of oxygen, for instance, reduced atmospheric pressure, heart failure, foetal life and the newborn state. All these states are associated with the preponderance of anaerobic over aerobic metabolism in the tissues, and with the increase of blood pyruvic acid.

The augmentation of pyruvate may be explained by its decreased breakdown, increased formation or decreased excretion. The parallelism which is found in the changes of pyruvate in the blood (*Gonzales* and *Gardner* 1957) and the urine (*Tallqvist* 1952) during the period subsequent to birth seems to exclude the last possibility. *Gonzales* and *Gardner* (1957) think that in the foetus and the newborn the elevated pyruvate concentration is due to increased production and normal or diminished utilization of pyruvic acid. The increased formation of the latter in anoxia has been borne out by the *in vitro* experiments of

Ikeziri (1956) and *Thorn* et al. (1957), who incubated bone marrow and certain other organs at low oxygen tension. Altitude or low barometric pressure, *per se*, has no apparent effect (*Friedemann* et al. 1949).

Pyruvic acid, which may originate from sources outside the blood as well as from the erythrocytes, is normally formed in intracellular anaerobic glycolysis. The glycolytic rate of reticulocytes is estimated as 2 to 6 times as great as that of mature red cells (*Selwyn* and *Dacie* 1954, *Hollingsworth* 1955). This tallies with that for the bone marrow cells (*Ikeziri* 1956). Glycolysis increases markedly in anaerobic conditions and is attributed to excessive utilization of carbohydrates in the tissues. However, no systematic change in the amount of red cell glucose is found during repeated bleedings (*Piha* 1956) although, as the present results show, the pyruvic acid concentration increases. On the other hand, *Vitacca* and *Pagano* (1955) established a correlation between the increase of pyruvic acid and that of glucose in carbon monoxide intoxication.

Other metabolic pathways leading to the production of pyruvic acid are, of course, available. *Markees* and *Meyer* (1949) found that administration of amino acids to human subjects resulted in an elevation of pyruvic acid through desamination reactions. Since the amino acid concentration in the red cells is increased in response to blood loss (*Piha* 1956) part of the red cell pyruvate may be of this origin.

Under anaerobic conditions the pyruvic acid formed *via* glycolysis undergoes reduction by lactic dehydrogenase to lactic acid, the reaction being reversible. Oxidatively, especially under aerobic condition, pyruvic acid is destroyed by pyruvic dehydrogenase, yielding acetic acid, which undergoes oxidation through the tricarboxylic acid cycle.

Pyruvic dehydrogenase, which, to act, needs an organized group of coenzymes, is responsible for the reaction:

$$\text{(1) Pyruvic acid} + \text{CoA} + \text{DPN} \xrightarrow[\text{lipoic acid, } Mg^{++}]{\text{diphosphothiamine}} \text{Acetyl-CoA} + CO_2 + \text{DPN} \cdot H_2$$

The oxidative degradation of pyruvic acid is prevented if any of the factors is deficient or if inhibitory agents exert an effect. In either case there would be an accumulation of pyruvic acid. Should the prosthetic group of the enzyme, diphosphothiamine (cocarboxylase), be lacking, the initial step of the reaction, the formation of the aldehyde-diphosphothiamine complex, would not proceed. *Greig* and *Govier* (1943) found a deficiency of cocarboxylase in the muscular tissue in haemorrhagic dogs and in dogs subjected to anoxic anoxia (tissue anaerobiosis). However, raised pyruvate values in haemolytic states are not associated with a deficiency of thiamine (*Allibone* 1948). On the other hand, its pyrophosphoric ester cocarboxylase depresses the increased pyruvate values in the blood to a normal level in decompensation of the heart (*Dalla Torre* 1952, *Lasch* 1953, *Greif, Hochegger* and *Bermani* 1957). Hence, in states which result in reduction of oxygen tension the phosphorylation of thiamine seems to be diturbed. This agrees with the statement of *Peters* (1948) that the pyruvic oxidase system is very sensitive to oxygen deficiency and with the findings that in experiments with various tissue slices under anaerobic condition the synthesis of diphosphothiamine is much decreased (*Ochoa* 1939, *Govier* and *Greig* 1943).

In anoxia (*Thorn* et al. 1957) and in haemorrhagic shock (*LePage* 1946, *Engel* 1952, p. 386) the resynthesis of adenosine triphosphate (ATP) in the tissues is decreased, and this would result in failure of phosphorylation of thiamine (thiamine + ATP → diphosphothiamine + AMP) followed by accumulation of pyruvic acid. On the other hand, aerobiosis increases ATP in avian red cells (*Engelhardt* 1932). It is known that in reticulocytes, in contrast to erythrocytes, there prevails an aerobic condition with aerobic metabolism (*Komatsu* 1957, and others). A considerable increase in the content of ATP in the red cells has been observed in haemorrhagic anaemia (*Wolff* and *de Lavergne* 1948, *Rottino, Hoffman* and *Albaum* 1952). In reticulocytes its content is 2—3 times higher than in the mature red cells (*Granick* 1949). Thus, the synthesis of diphosphothiamine and the oxidative breakdown of pyruvic acid in reticulocytes is rendered possible.

Because pyruvic dehydrogenase requires several substances for its activity it is very vulnerable. The oxidative degradation of pyruvic acid is blocked, for instance, by the lack of lipoic acid and the components of CoA, accumulation of pyruvate again being the result (cf. *Pilgrim, Axelrod* and *Elvehjem* 1942, *Redetzki, Bloedorn* and *Bansi* 1956).

To conclude, the pyruvic acid augmentation in the *plasma* during haemorrhagic anaemia may be due partly to increased formation occurring everywhere outside the blood and partly to decreased utilization. The latter may be attributed to a failure of the tissues to phosphorylate thiamine or to a lack of other necessary coenzymes. By contrast, in the *red cells* and particularly the reticulocytes, the augmentation of pyruvic acid might be explained (1) by its passage from the plasma into the red cells across the cell membrane and (2) by increased intracellular formation *via* glycolysis or through other metabolic pathways.

5. Role of pyruvic acid in blood regeneration.

Does the rise of pyruvate in the red cells play any role in general cell metabolism and particularly in haemoglobin synthesis or is it only a waste product without any specific significance? As yet no convincing interpretation can be offered.

In the mammalian red cells glycolysis is the main source of the energy required for maintenance of the cell. No evidence of the utilization of pyruvate in the mature erythrocytes has been found (*Rubinstein* and *Denstedt* 1953). In aerobic conditions, such as prevail in the reticulocytes, energy may also be derived from pyruvic acid. In the reticulocytes there is a greater need of energy for synthetic activity and for transport of ions across the cell membrane.

The red cells, which are capable of respiration, seem to contain the enzymes involved in the tricarboxylic acid cycle (nucleated erythrocytes: *Rubinstein* and *Denstedt* 1953, *Dajani* and *Orten* 1958; reticulocytes: *Behrendt* 1957, p. 188, *Komatsu* 1957). The same applies to the bone marrow cells, in which

pyruvic acid formation is parallel to oxygen consumption (*Ikeziri* 1955). Pyruvic acid is linked *via* acetyl-CoA to the above cycle, in which the latter is converted to succinate.

(2) Acetyl-CoA + oxaloacetic acid → citrate → succinate

It may be assumed that in the reticulocytes pyruvate is a source of the »active« acetate required for the synthesis of succinate. This view is supported by the experiments of *Rubinstein* and *Denstedt* (1953), who deduced that in avian red cells pyruvate is utilized in the synthesis of citrate. The utilization of citric acid-1,5-C^{14} for porphyrin formation in avian red cell haemolysate is evidenced (*Wriston, Lack* and *Shemin* 1955). This agrees with the conclusion of *Dajani* and *Orten* (1958) that the tricarboxylic acid cycle may serve as a source of succinate for the formation of porphyrins in nucleated erythrocytes.

The condensation product of the »active« succinate and glycine is δ-amino-β-adipic acid, which is readily converted to δ-aminolaevulic acid (*Shemin, Russell* and *Abramsky* 1955).

(3) Succinic acid + CoA + DPN → succinyl-CoA
(4) Succinyl-CoA + glycine → δ-aminolaevulic acid

The latter is the immediate precursor of porphyrin (*Neuberger* and *Scott* 1953, *Shemin* and *Russel* 1953, *Schiffmann* and *Shemin* 1957). *Granick* (1958) showed very recently that the synthesis of δ-aminolaevulic acid by chicken red cells requires members of the citric acid cycle.

Since succinyl-CoA is a potential source of porphyrin, pyruvic acid may be regarded as a link between the metabolism of carbohydrates (proteins, fats) and that of porphyrins. Raised pyruvate values have been recorded in several states mentioned above in which blood regeneration and haemoglobin production are also known to be increased. In spite of the entrance of pyruvic acid into the tricarboxylic acid cycle, its level in the red cells is maintained high. This is explained by the passage of pyruvic acid from the plasma and by the enhanced glycolysis.

The interval between the first bleeding and the appearance of pyruvate-rich red cells is approximately the same, about three

days, as is required for activation of the bone marrow and for the development of a new cell population to the reticulocyte stage (*Piha* 1956). The synthesis of haemoglobin still continues in the metabolically active haemoglobin-deficient reticulocytes released from the bone marrow in response to severe anaemia (*London, Shemin* and *Rittenberg* 1950, and others). The present work shows a correlation between the elevated pyruvate concentration of the red cells and the reticulocytosis which is considered to be a sign of increased blood regeneration. It has also been ascertained that haemoglobin and red cell content (*Findlay* 1946) and blood pyruvate concentration (*Schreier* and *Dankwart* 1951) increase considerably within the first few hours after birth both decreasing after the initial post-natal increase during the first weeks of life.

From the foregoing it might be assumed that the significance of red cell pyruvic acid during blood regeneration lies in its coupling into reaction sequences which are responsible for the increased formation of haem. *Schreier* (1956, p. 42) is of the opinion that in the newborn the raised pyruvate concentration may be attributed to a foetal haemoglobin which delivers oxygen slowly.

6. Sources of error.

Certain sources of error affect the pyruvic acid values obtained. A biological (positive) error is introduced during the regeneration period by the withdrawal of blood samples, which has a slight anaemizing effect. Besides this, the secretion of epinephrine and the diet (*Friedemann* et al. 1949) affect the blood pyruvate level.

Some error may be attributed to the determination technique, in which α-ketoglutaric acid (*Slavic* and *Michalec* 1949), acetone and acetoacetic acid are sources of error. Turbidity or opalescence of the plasma which may occur on the 5th—8th day of the experiment, has a disturbing (positive) effect on plasma protein values determined by the biuret reaction. The reliability of results which have been expressed as a percentage of the

normal depends entirely on the accuracy of the initial value, and here a source of error may exist.

Storage of plasma and red cells increases the content of pyruvic acid (*Blanchaer* and *Baldwin* 1953). The glycolytic processes are very much depressed during storage at 4°C, whilst at 37°C these may proceed, especially in red cells (*Henley, Wiggins* and *Pollard* 1956), at the *in vivo* rate for a certain time. In protein-free filtrates at 2°C under toluene the pyruvic acid concentration remains almost unchanged for several days.

VI. Summary.

The aim of the present experiments was to throw light on the pyruvic acid metabolism in the plasma and the red cells during increased blood regeneration due to repeated bleedings. The anaemia produced was simultaneously combined with hypo- and hyperproteinaemic states.

1. The rabbits were rendered »anaemic-hypoproteinaemic», »anaemic-normoproteinaemic», »anaemic-hyperproteinaemic» and »anaemic» (Groups I, II, III and IV) by repeated bleedings and by repeated transfusions of 0.9 per cent $NaCl^-$ or plasma to the circulation. The anaemia produced varied between 35 and 65 per cent of the initial haemoglobin value. The hypoproteinaemia was approx. 83 per cent and hyperproteinaemia 112 per cent of the initial plasma protein level.

2. The normal pyruvic acid concentration averaged in comparable experiments is 2.65 mg/100 ml (S.D. = 0.92) for plasma and 1.84 mg/100 ml (S.D. = 0.33) for red cells. The average [Red cell pyruvate] / [Plasma pyruvate] ratio in the normal rabbit is 0.69 (S.D. = 0.12).

3. In the above corresponding groups a conspicuous increase of pyruvic acid in the plasma (100, 160, 70 and 34 per cent) and in the red cells (97, 140, 98 and 66 per cent) in response to blood

loss was observed. The steepest rise of pyruvic acid concentration both in the plasma and the red cells was established on the 3rd to 5th day of the experiment. Correspondingly, in each group repeated bleedings resulted in an increase of the [Red cell pyruvate] / [Plasma pyruvate] ratio.

4. The increase of pyruvic acid in the plasma and the red cells is correlated with the degree of anaemia. The lower the level to which the haemoglobin was reduced, the greater the increase of pyruvic acid concentration was found to be.

5. The variable plasma protein level, concurrent with the haemorrhagic anaemia, seems to exert a slight effect on the plasma pyruvic acid concentration, but does not noticeably affect the pyruvate changes in the red cells. A high plasma protein level appears to reduce the rise of plasma pyruvic acid.

6. The red cell pyruvic acid gradually increases with the increasing number of reticulocytes. Likewise, the concentration ratio [Red cell pyruvate] / [Plasma pyruvate] is correlated with the progressive reticulocytosis. As the number of reticulocytes rises from 4 to 21—28 per cent the ratio increases correspondingly from 0.68 to 1.1.

7. Normally the red cell membrane is freely permeable to pyruvic acid, whilst the reticulocytes seem to possess the ability to concentrate pyruvic acid or to maintain it at a high level. It is concluded that the pyruvic acid increase in the red cells is for the most part due to the increased concentration of plasma pyruvic acid, derived mainly from cells outside the blood, and to a smaller extent of intracellular origin.

8. The causation of the augmentation of pyruvic acid is discussed. The relationship between the increase of blood pyruvic acid and states involving a deficient supply of oxygen is considered.

9. The significance of pyruvic acid in the red cells is discussed. It is suggested that during blood regeneration pyruvic

acid enters *via* acetyl-CoA into the reaction sequences (tricarboxylic acid cycle and succinate-glycine cycle) which are responsible for the formation of haem.

Acknowledgements.

I wish to express my gratitude to Professor *Yrjö Reenpää*, M.D., the Head of the Institute of Physiology, University of Helsinki, for his help to publish this investigation.

To Professor *Eeva Jalavisto*, M.D., and Docent *M. J. Karvonen*, M.D., Ph.D. I am obliged for fruitful discussions and criticism.

The technical assistance of *S. Lindholm*, M.D., *J. Lähdevirta*, M.B., and *A. Raina*, M.B., in the experimental work is gratefully acknowledged.

For the revision of my English manuscript I wish to thank Mr *R. C. Holloway*, M.Sc., and Mrs *Jean Margaret Perttunen*, B.Sc.

This work has been supported by grants from the Finnish State Committee for Natural Sciences and from the Rockefeller Foundation.

Helsinki, in May 1958.

S. P.

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